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High incidence of sperm dysfunction in a varicocele infertile man: case report

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ABSTRACT

Studies indicate abnormal semen indicators among varicocele infertile men can be reversed to normal status after surgical repair. While semen indicators and DNA damage of sperms are reported frequently, sperm function tests are rarely performed to assess the functional status of sperms among these individuals. We report a 35-year-old male with 4 years of primary infertility who otherwise has a normal sexual life. Various analyses performed revealed the interplay of multiple abnormalities leading to the observed phenotype. The individual was diagnosed with severe sperm defects, bilateral varicocele (grade II) and endocrinopathy. The percentage of functionally normal sperms were found to be 24% for hypo-osmotic swelling, 28% for acrosome reaction and 21% for nuclear chromatin decondensation test. Cytogenetic analyses showed normal karyotype and sequence-tagged-site markers based PCR showed no deletions involving key candidate genes of the Y chromosome. A thorough investigation of infertile subjects and simple diagnostic tests are essential to detect the treatable defects, in general as well as severe infertile cases, which can improve the chances of normal conception or the success rates of *in-vitro* fertilization and intracytoplasmic sperm injection.

1. Introduction

Varicocele is the pathological dilation of the pampiniform plexus of veins (network of many small veins draining the scrotum) of the spermatic cord. The incidence of varicocele is about 35%-50% in men with primary infertility and is as high as 70%-80% in men with secondary infertility (inability to conceive after giving birth to at least one child)[1,2]. The pathophysiology of varicocele induced male infertility includes hypoxia, testicular venous hypertension, elevated testicular temperature, stasis, *etc*., and these affect the normal testicular function, causing a decline in semen parameters like count, motility, morphology, *etc*[3]. Apart from these oxidative stress through excess production of reactive oxygen species (ROS) and reactive nitrogen species, DNA damage, reduced total antioxidant capacity, *etc*. observed in these individuals are reported to affect spermatogenesis[4].

Here, we report a varicocele infertile individual with severe sperm function defects greater than that observed in the infertile men, along with endocrine disruption, but without a genetic predisposition.

2. Case report

A 35-year-old male with 4 years of primary infertility was referred for diagnosis. The couple had secondary consanguinity of uncle-niece union. The individual was diagnosed with severe sperm defects, bilateral Varicocele (grade II) and endocrinopathy.

Semen collection, physical and microscopic examination were carried out as per the standard WHO protocol[5]. Physical examination revealed testicular volume to be 13 mL each (normal 15-20 mL). Semen analyses revealed severe morphological defects of the sperms (95%), low vitality (32%) and low sperm count of 2.1×10^6 sperms/mL. Hormone analysis showed increased levels of follicle-stimulating hormone and luteinizing hormone but low levels

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of testosterone. The obtained values in comparison with the normal range are depicted in Table 1. The profile was an indication of failure of the negative feedback mediated by the testicular products testosterone and inhibin.

To assess the plasma membrane integrity, 10 μL of liquefied semen sample was mixed with 50 μL of hypo-osmotic swelling (HOS) solution and incubated at 37 °C for 15 min. After the incubation period, 5 μL of stop solution was added. A drop of the incubation mixture was placed on a clean glass slide and observed under the microscope[6].

Acrosome status was determined by incubating a mixture of 10 μL of liquefied semen sample in phosphate buffered saline D-glucose (1:20, v/v) at 37 °C for 15 min. A fraction of the incubated mixture was then smeared onto a gelatine coated slide followed by incubation at 50 °C for 30 min in a moist humid chamber. The slides were then air dried and observed under the microscope[7].

Nuclear chromatin decondensation (NCD) test was performed by the method described by Gopalkrishnan[7]. First, 10 μL of liquefied semen sample was diluted with 50 μL of ethylenediaminetetraacetic acid and sodium dodecyl sulfate mixture. The mixture was then incubated with equal volume of glutaraldehyde/borate buffer at 37 °C for 15 min. Finally, 10 μL of the incubated mixture were then placed on a clean glass slide and examined under a microscope.

Sperm function tests revealed low values for HOS test (Figure 1), acrosome reaction (AR) test (Figure 2) and NCD test (Figure 3). The percentages of functionally normal sperms were found be 24%, 28% and 21% for HOS, AR and NCD tests, respectively, against the normal values of 70%, 60% and 50% (low reference value).

Y chromosomal microdeletions screening was carried out employing sequence-tagged-site markers specific to the key candidate genes, namely, deleted in azoospermia (*DAZ*), RNA binding motif Y 1 (*RBMY*1) and ubiquitin specific protease 9 Y (*USP9Y*). The markers employed were sY254 and sY255 for *DAZ* gene, sY627 for *RBMY*1 gene and sY1316 for *USP9Y* gene. The primer details and target sequences are provided in Table 2. The reaction was carried out in 25 μL reaction volume in a corbett themocycler with following thermal profile- initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The products were then separated on 2% agarose gel by electrophoresis and visualized under UV trans-illuminator.

Y chromosomal microdeletions screening employing sequence-tagged-site markers specific to the key candidate genes, namely, *DAZ* (sY254 and sY255), *RBMY*1 (sY627) and *USP9Y* (sY1316), also confirmed lack of any major deletion event.

Peripheral blood was cultured in RPMI-1640 medium with 15% (v/v) fetal bovine serum and phytohaemagglutinin at 37 °C for 72 h. Cultures were harvested using colcemid and hypotonic (5.6 g/L potassium chloride) solution followed by fixation with acetic acid and methanol (1:3, v/v). Slides were prepared by air-drying method. GTG banding was performed on freshly prepared slides using standard protocols. Fifty cells were analyzed. Chromosomal analysis revealed a normal male karyotype of 46, XY.

Fluorescence in situ hybridization was performed according to the manufacturer's instructions on the metaphase spreads of the patient by standard procedures. Commercially available alpha-DNA satellite probes for X and Y chromosome (DXZ1 and DYZ3, Poseidon probes from Kreatech, Netherlands) were used. Fluorescence in situ hybridization with SEP(X/Y) showed no sex chromosomal anomaly in this case (Figure 4).

Table 1
Normal range of hormone titer and obtained values of the individual.

Hormone	Observed value	Normal range
FSH (mIU/mL)	18.9	1.0-14.0
LH (mIU/mL)	12.5	0.5-5.6
Testosterone (ng/dL)	4.3	2.0-6.9

FSH, Follicle-stimulating hormone; LH, Luteinizing hormone.

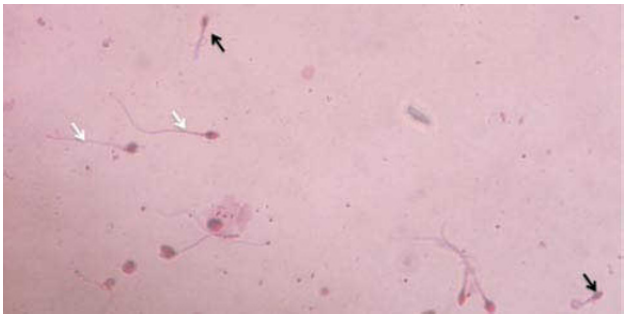

Figure 1. Hypo-osmotic swelling test.
Black arrowheads indicate the normal sperms exhibiting tail coiling which represents sperm with good plasma membrane integrity, while white arrowheads highlight the abnormal sperms with poor or no coiling due to lack of intact membrane.

Table 2
List of the details of the sequence-tagged-site (STS) markers.

STS	Location	PCR product size (bp)	Forward primer	Reverse primer
sY254	*DAZ* exon 3	380	5′-GGGTGTTACCAGAAGGCAAA-3′	5′-GAACCGTATCTACCAAAGCAGC-3′
sY255	*DAZ* intron	126	5′-GTTACAGGATTCGGCGTGAT-3′	5′-CTCGTCATGTGCAGCCAC-3′
sY627	*RBMY*1 exon 12	104	5′-GCACCTGCCACGCATATAGT-3′	5′-GCAAACATGCTCACGATCAC-3′
sY1316	*USP9Y* exon 26	463	5′-AAGGCAGGTCTGATGCATGT-3′	5′-AAAGAAAGCTGCCTCATAGCA-3′

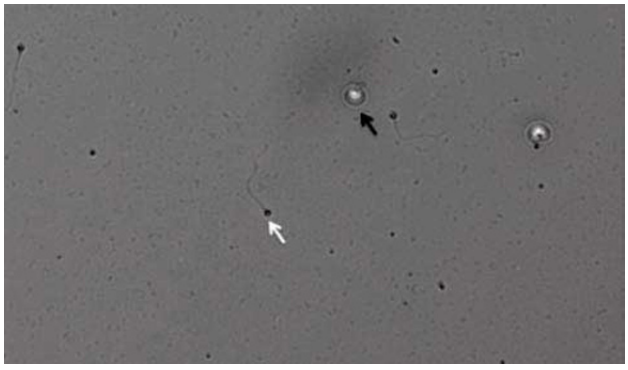

Figure 2. Acrosome reaction test.
Black arrowhead points to the halo formation in the head region which is indicative of normal acrosome, while the white arrowhead indicates those sperms with abnormal acrosome.

Figure 3. Nuclear chromatin decondensation test.
The white arrows highlight the enlargement of the sperm head indicating the ability of chromatin decondensation, whereas the black arrowheads mark the sperms that lack enlarged head indicating abnormalities associated with the sperm DNA packaging rendering them unable to decondense the chromatin.

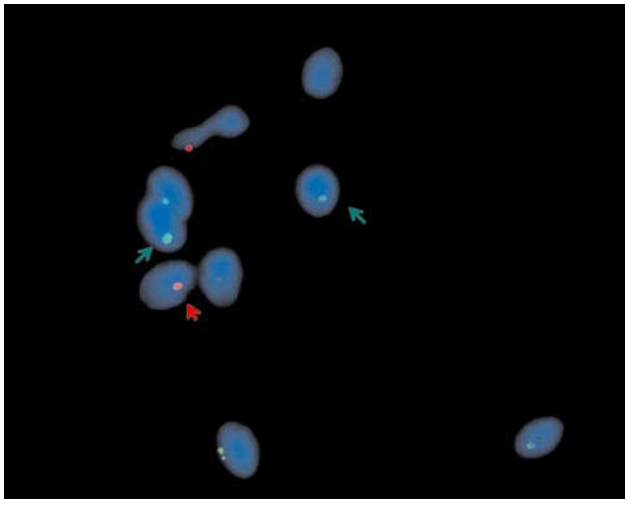

Figure 4. Fluorescence in situ hybridization on the sperm samples of the individual with SE X (DXZ1)/ SE Y (DYZ3) probe.
The green signals indicate the X-bearing sperms and the red signals indicate the Y-bearing sperms.

3. Discussion

It is reported in the literature that abnormal sperm parameters among varicocele infertile men can be corrected after surgery[8]. In majority of cases, DNA damage, oxidative stress and altered semen parameters are reported. In our work, we report a varicolele patient and assessed the sperm function test.

Semen analysis enabled to document the infertile condition and any prominent diagnosis was distinctly unclear at this level. Semen parameters like count and motility cannot alone predict the ability of the spermatozoa to perform the prerequisite cellular processes essential to fertilize the oocyte. In this concern, sperm function tests are essential to evaluate the defects, if any, present in acrosome, plasma membrane functions and chromatin packaging[9]. Sperm head region contain a cap like structure called as acrosome that contains enzymes and binding sites for attachment to the egg. Exocytosis of sperm acrosomal contents is an important prerequisite for penetreation of zona pellucida as it facilitates the downstream processes required for nuclear fusion. The AR test involves staining the sperm acrosome cap and then stimulating the sperms to release their caps. Absence of acrosome reaction implies poor prognosis for fertilization. Assisted reproductive techniques like intra uterine insemination (IUI) and *in-vitro* fertilization (IVF) can result in failure owing to this specific defect. Men whose sperm does not undergo the AR in this test have very poor fertilization rates at IVF[10]. It is clinically important to know whether the immotile spermatazoa are alive or dead. Hence, vitality results should be assessed. The presence of a large proportion of vital but immotile cells may be indicative of structural defects in the flagellum; a high percentage of immotile and non-viable cells (necrozoospermia) may indicate epididymal pathology. A semen sample is considered normal if 58% or more of the sperm cells are alive[5]. In our case, we found that the percentages of vital sperms were low recording 32%.

The HOS test help in assessing the diagnosis and the management of male infertility. This test is based on the semi-permeability of the intact cell membrane which causes the spermatozoa to swell. Sperm swimming through cervical mucus requires a different pattern as compared to that observed in semen under the microscope and only motile sperms will have a chance to fertilize the egg. Sperm membrane integrity is vital to regulate ion and water flux which is essential for normal motility pattern. Defects in the membrane integrity can lead to poor motility pattern which might lead to failure of IUI and IVF process[10]. Apart from acrosome and membrane intactness, abnormal genetic component can have severe impact on fertilization and early

development. Decondensation of the nucleus and formation of the pronucleus are the penultimate steps in the sequence of events leading to fertilization. Any abnormalities in the nuclear decondensation can lead to failure of fertilization[11]. The scores of sperm function tests in the cases analyzed serve to reveal the risk for following Assisted reproductive techniques like IUI, IVF and intracytoplasmic sperm injection as the success rates will be affected by the poor functional status of the sperms, not to mention the costs involved.

Transrectal ultrasound scanning and ultrasound scanning were used to document abnormalities in the testis and accessory reproductive glands. The individual had bilateral varicocele with grade II severity. Varicocele has been demonstrated to cause nuclear DNA damage, raised levels of ROS, and increase in apoptosis[4]. The poor NCD score obtained might be a manifestation of varicocele by increasing the ROS levels.

To look for possible genetic abnormalities, karyotyping, Y chromosomal microdeletions screening and sperm fluorescence in situ hybridization analysis were performed. All the three procedures demonstrated negative results clearly ruling out possible genetic etiology. This evades the need for genetic counselling for the individual reported in the study.

The case highlights the findings made through sperm function tests. This diagnosis can help prevent unnecessary, expensive treatment options like IVF and intracytoplasmic sperm injection, prematurely without taking appropriate measures to improve the sperm quality through medical intervention and lifestyle modification.

Conflict of interest statement

We declare that we have no conflict of interest.

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